Engineering bacteria for the degradation of halopropanes

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Microbial strategies for the removal of environmental pollutants from waste streams or contaminated sites can provide an attractive alternative to traditional methods such as incineration or disposal in land fills, but the applicability of bioremediation is often limited by the lack of suitable organisms. An important group of contaminants for which efficient treatment methods are needed are halogenated aliphatic compounds since they may adversely affect human health and pose a threat to ecosystems. Some of these compounds are generated by naturally occurring biotic and abiotic processes in the oceans and the atmosphere (26). However, the use of halogen–based chemistry in industrial synthesis over the past 100 years has generated many new halogenated aliphatic compounds, ranging in application from solvents and polymers to degreasing agents and biocides (21). As a result of their intended use, accidental release, or due to improper waste disposal policies, vast amounts of halogenated aliphatic compounds have been released into the environment.

One consequence of the appearance of new synthetic organic chemicals in the environment has been the remarkably rapid evolution of microorganisms with new catabolic activities. For example, the extensive use of the herbicide atrazine has provided the selective pressure for the evolution of a new catabolic pathway for the degradation of this chemical. It has been suggested that the atrazine degradative pathway has been assembled recently, over a period of decades (50, 82). Due to their metabolic versatility and evolutionary potential, microorganisms are able to utilize an amazing spectrum of noxious compounds, including halogenated chemicals such as 1,2-dichloroethane (33) and vinyl chloride (27), and pesticides such as γ-hexachlorocyclohexane (lindane) (32) and 1,2-dibromoethane (65). Despite the fact that a variety of microorganisms can utilize these chemicals as growth substrate, many halogenated aliphatic compounds persist in the environment, which emphasizes the inadequacy of the current catabolic capacity to deal with all recalcitrant pollutants. Gene technology, combined with the extensive knowledge base of the microbiology, biochemistry, and genetics of catabolic pathways, has been proposed to enable the engineering of new or improved catabolic activities for such pollutants.

Over the past decades research on the degradation of short-chain haloaliphatics has been focused mainly on chloromethanes, chloroethanes, and chloroethenes, since these compounds are produced industrially in large amounts and represent a widely recognized class of priority pollutants (21). Biodegradation of halogenated propanes has drawn less attention, although halopropanes such as 1,2-dichloropropane (90, 99), 1,2,3-trichloropropane (TCP) (91, 92, 99), and 1,2-dibromo-3-chloropropane (3), also form an important group of toxic and recalcitrant pollutants, in part due to their chemical and biological stability. Thus far, no pure cultures of bacteria that are capable of aerobic growth on these compounds have been isolated by classical adaptation and enrichment techniques. Nevertheless, it is possible to design pathways on paper which bacteria could use for growing on such chemicals. The engineering of microorganisms for the degradation of recalcitrant halogenated aliphatic compounds is therefore a challenging goal. In this chapter I describe current knowledge of microorganisms and catabolic enzymes involved in the aerobic degradation of

C3-halogenated aliphatics, with an emphasis on halopropanes. In addition, recent advances in the engineering of bacteria and catabolic enzymes for bioremediation will be highlighted.

C3-halogenated aliphatics

Table 1 summarizes a number of relevant C3-haloaliphatics which are of environmental and industrial importance. In terms of industrial production epichlorohydrin is the most important member of the C3-haloaliphatics, with a global production of about 700,000 ton/year. Epichlorohydrin is a versatile building block for the synthesis of epoxy resins, rubbers, adhesives, agrochemicals, and pharmaceuticals. In particular, optically active compounds have broad potential for synthesis (36). Commercial epoxide production occurs

Table 1. Industrial and agricultural uses of various C3-haloaliphatic compounds.

Compound	Uses[a]
1-Chloropropane	Solvent
1-Bromopropane	Intermediate in chemical synthesis of pharmaceuticals
1,2-Dichloropropane	Nematicidic soil fumigant, solvent, insecticide for stored grain
1,3-Dichloropropane	Alkylating agent, ring-forming agent, polymerization catalyst in the synthesis of organic chemicals
1,2,3-Trichloropropane	Solvent, extractive agent, chemical intermediate in the production of polysulfone liquid polymers and dichloropropene, cross-linking agent in the synthesis of polysulfides
1,2-Dibromo-3-chloropropane	Nematicidic soil fumigant of citrus crops
Epichlorohydrin	Intermediate in the production of epoxy resins, synthetic glycerin, elastomers
1,3-Dichloro-2-propanol 2,3-Dichloro-1-propanol	Precursor optically active compounds,
1,3-Dichloropropene	Nematicidic soil fumigant

[a] Sources: Babich et al., (3); Belkin, (4); van Agteren et al., (93); Tesoriero et al., (90); National Priority fact sheet, (91)

mainly via the chlorohydrin process with chloropropanols as intermediates. Aqueous effluent emerging from epoxide production processes for the manufacture of propylene oxide or epichlorohydrin, for example, can contain considerable amounts of undesirable chloroaliphatic compounds, such as 1,2-dichloropropane and 1,2,3-trichloropropane, respectively, of which the removal is very expensive (63, 88).

A number C3-haloaliphatics have been used as constituents of soil fumigant formulations. The nematocides Shell D-D and Telone II comprise a mixture of

1,3-dichloropropene and 1,2-dichloropropane and have been used worldwide in agriculture. In addition, TCP was often found as a significant contaminant of these fumigant formulations. The extensive application of these soil fumigants has led to pollution of soils and waters in the Netherlands and United States (90, 99). The highly persistent brominated organochlorine compound 1,2-dibromo-3-chloropropane was used as a nematocide in citrus crops in the United States. It is 8 to 16 times as effective as the D-D mixture, and has been produced for agricultural use since 1955. In 1979, the United States Environmental Protection Agency banned almost all agricultural uses of 1,2-dibromo-3-chloropropane, which was triggered by a report on sterility in workers who had handled 1,2-dibromo-3-chloropropane in a pesticide division of a chemical plant in California. However, 1,2-dibromo-3-chloropropane is still detected as a groundwater contaminant in the United States, illustrating the persistence of this chemical (3).

Because of the chemical and biological stability of halopropanes, and to a lesser extent also C3-epoxides and halopropanols, these compounds can be widely distributed in the environment via water and air. Although these chemicals are often not toxic at the concentrations found in water and soil, they tend to accumulate in the tissues of higher animals and plants. Due to bioaccumulation deleterious effects may occur on organisms at or near the top of the food chain. The toxicity of these chemicals has been documented from surveys of employees handling these compounds and by tests with different laboratory animals (3, 28, 45). As a result they are now being recognized as potentially mutagenic, carcinogenic, and/or toxic to the liver, kidney, and to the nervous and reproductive system.

Causes of recalcitrance

Many halogenated aliphatics are slowly removed in the environment. Some obnoxious cases of persistent chemicals include 1,1-dichloroethane, 1,1,1-trichloroethane, the chlorinated ethylenes *cis*- and *trans*-1,2-dichloroethene, trichloroethene, and tetrachloroethene, 1,2-dichloropropane and 1,2,3-trichloropropane. From a thermodynamic point of view, aerobic degradation of halogenated aliphatics should yield sufficient energy to sustain bacterial growth. However, due to the presence of the carbon-halogen bond these compounds are often too far from the mainstream of catabolic pathways to be used as a substrate. Catabolic enzymes and regulatory proteins of existing metabolic routes do not properly recognize these xenobiotics, yielding insufficient flux of energy and intermediates that can be used for biosynthetic processes. This is not surprising, since microorganisms have been exposed to these chemicals only since about 100 years, a mere instant in evolutionary terms. For many xenobiotics, such as the highly toxic dioxins and PCBs, no effective degradative routes have been described. Often degradation of xenobiotics is incomplete or very slow, which may cause accumulation of reactive intermediates that destabilize microbial communities. For example, the 1,2-dichloroethane (DCE) utilizing bacterium *X. autotrophicus* GJ10 was not able to utilize 1,2-dibromoethane (DBE) as a growth substrate, although the organism seems to posses all the necessary enzymes for the complete degradation of DBE. It appears that the accumulation of the highly reactive

bromoacetaldehyde, due to the absence of a sufficiently active aldehyde dehydrogenase, hampered utilization of DBE for growth by this bacterium (Fig. 1 A and B) (95).

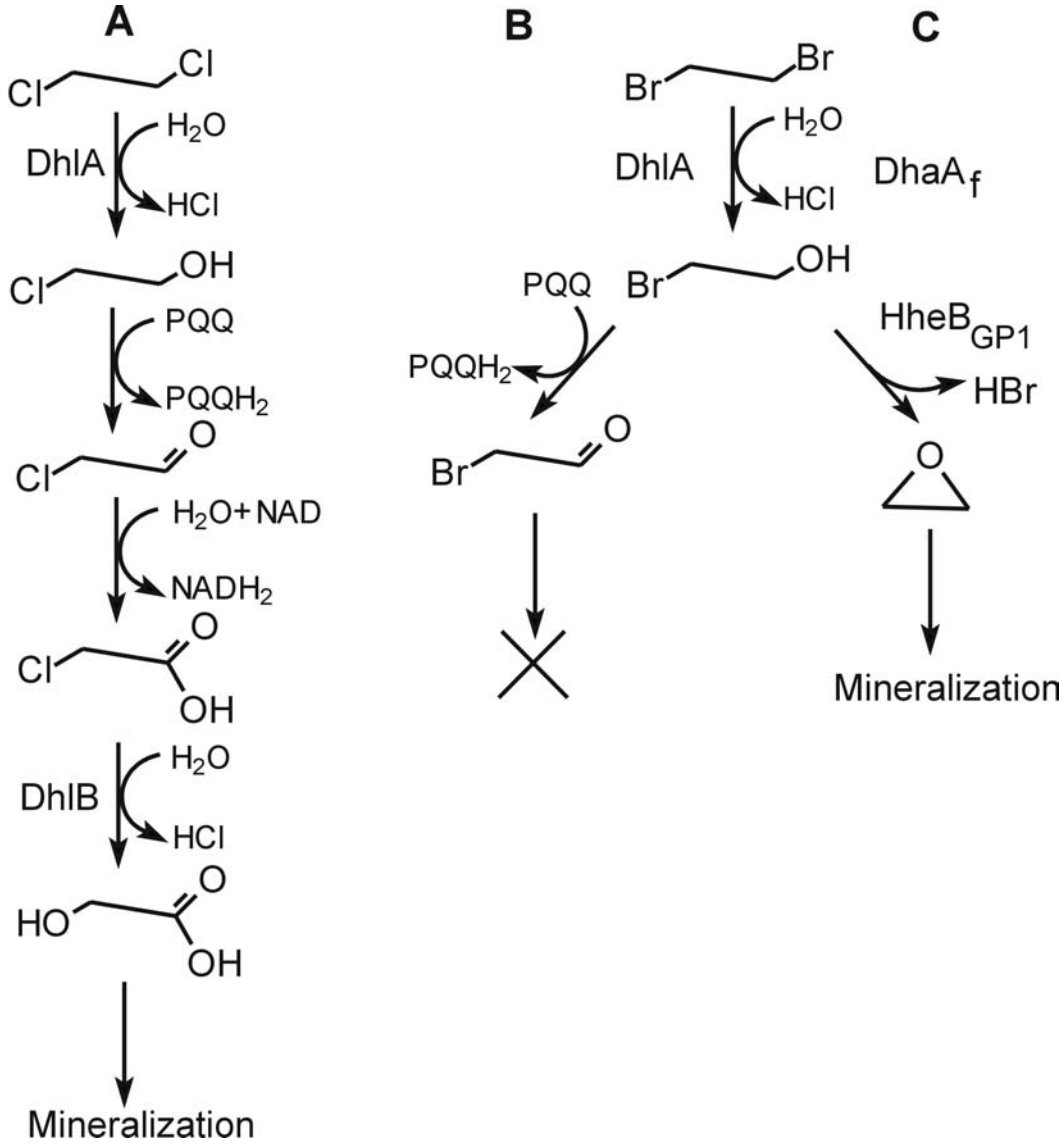

Fig. 1. (A) Proposed route of the metabolism of 1,2-dichloroethane in *X. autotrophicus* GJ10. (B) Proposed degradation route of 1,2-dibromoethane in *X. autotrophicus* GJ10. (C) Proposed route of the metabolism of 1,2-dibromoethane in *Mycobacterium* sp. strain GP1.

Consequently, 1,2-dibromethane was extremely toxic to the cells, and mutants that are resistant have lost the dehalogenase activity. Degradation of 1,2-dibromoethane therefore follows a different route. Recently, a 1,2-dibromoethane utilizing bacterium was isolated, *Mycobacterium* sp. strain GP1, which circumvents the formation of bromoacetaldehyde as a toxic intermediate by rapid conversion of 2-bromoethanol to ethylene oxide (Fig. 1C). The latter compound can serve as a growth substrate for strain GP1 (65).

Furthermore, misrouting may occur if organisms are exposed to incompatible mixtures of xenobiotics. For example, mixtures of chloro- and alkylaromatic pollutants which

are often found in industrial waste induce expression of both *ortho*- and *meta*-ring cleavage pathways and thereby cause misrouting of central catechol intermediates into unproductive pathways, perturbing productive metabolism, even of compounds that otherwise would be degradable (76). Thus, compounds that look similar at first sight may require very different routes.

A solely biochemical explanation is often not sufficient for understanding the observed persistence of halogenated compounds in the environment. The efficiency of degradation is also determined by various non-biological factors such as pH, temperature, salinity etc., since microorganisms usually operate within a fairly narrow range of physico-chemical conditions (75). Furthermore, microorganisms that in principle could derive energy from the degradation of halogenated compounds will not grow if the concentration of these compounds is below a certain threshold that is needed for the generation of new biomass. In that case, there will be no stimulation of growth of organisms that posses the required enzymes and the population of degraders will not increase. On the other hand, at high concentration direct toxic effects of an apolar chlorinated hydrocarbon can decrease the survival of degradative organisms. The limited bioavailability of apolar and poorly water-soluble compounds also can contribute to the persistence of xenobiotics in the environment. Many compounds tend to adsorb onto soil particles or diffuse into clay structures and thereby become less available to microorganisms (1). Thus, understanding the bottlenecks of xenobiotic degradation requires a combined approach. The biochemical causes of the persistence of halogenated compounds may possibly be overcome by optimization of enzymes and metabolic pathways in organisms relevant for biodegradation.

Exploring and analysis of the natural diversity for the degradation of C3-haloaliphatics

The use of microorganisms to degrade synthetic halogenated aliphatic compounds is an emerging technology (86). The success of this technology will in part depend on our ability to select or engineer microorganisms that can degrade compounds that are currently not biodegradable. For this, the naturally occurring microbial activities are the starting point (Table 2).

Microbial degradation of halogenated aliphatics

Aerobic microbial degradation processes can roughly be divided into two classes: cometabolic conversions and utilization of the organic compound as a growth substrate (93). Mineralization of halogenated aliphatic compounds, which always occurs if a compound support growth, is the most favorable way to eliminate environmental pollutants. An increasing number of aerobic microorganisms have been isolated that can utilize haloaliphatics as a growth substrate. Some remarkable examples are bacteria that can grow on dichloromethane, 1,2-dichloroethane, and vinyl chloride, or on pesticides such as 1,2-dibromoethane, all of which are produced in large quantities by the chemical industry.

Table 2. Examples of microbial dehalogenation of C3-haloaliphatic compounds

Substrate	Organism	Mode of dehalogenation activity	Culture	Ref.
1-Chloropropane	*Xanthobacter autotrophicus* GJ10	hydrolytic	P	(33)
	Rhodococcus rhodochrous NCIMB 13064	hydrolytic	P	(17)
	Methylosinus trichosporium OB3b	oxygenolytic	C	(83)
1,2-Dichloropropane	*Xanthobacter autotrophicus* GJ10	hydrolytic	D	
	Methylosinus trichosporium OB3b	oxygenolytic	C	(61)
	Nitrosomonas europaea	oxygenolytic	C	(100)
1,3-Dichloropropane	*Xanthobacter autotrophicus* GJ10	hydrolytic	P	(33)
	Rhodococcus sp. strain M15-3	hydrolytic	D	(107)
1,2,3-Trichloropropane	Strain m2c-3	hydrolytic	D	(107)
1,2-Dibromo-3-chloropropane	*Nitrosomonas europaea*	oxygenolytic	C	(100)
1,3-Dichloro-2-propanol	*Agrobacterium radiobacter* strain AD1			(105)
	Arthrobacter strain AD2			
	Coryneform bacteria strain AD3			
	Flavobacterium sp.	intramolecular	P	(10)
	Corynebacterium sp. N-1074	substitution		
	Pseudomonas sp. strain OS-K-29			(38)
	Alcaligenes sp.			(36)
2,3-Dichloro-1-propanol	*Agrobacterium radiobacter* strain AD1			(6)
	Pseudomonas sp. strain OS-K-29	intramolecular	P	(38)
	Alcaligenes sp.	substitution		(36)
	Agrobacterium sp.			(19)
2,3-Dibromo-1-propanol	*Flavobacterium* sp.	Intramolecular substitution	P	(10)
1-Chloro-2-propanol	Strain AD18/AD19	unknown	M	Van den Wijngaard, unpublished results
1,3-Dichloropropene	*Pseudomonas cichorii* strain 170	hydrolytic	P	(66)

P, pure bacterial culture growing on the substrate; C, Cometabolic conversion by oxygenease producing bacteria; D, Cometabolic conversion by haloalkane dehalogenase producing bacteria; M, Mixed culture growing on the substrate.

Of the C3-halogenated aliphatics only a few mono- and dihalo-compounds are known to support microbial growth (Table 2). This includes organisms growing on epichlorohydrin, various halopropanols, and bacteria that degrade the soil fumigant 1,3-dichloropropene. Halogenated aliphatic hydrocarbons such as 1,2-dichloropropane (DCP), 1,2,3-trichloropropane (TCP), and 1,2-dibromo-3-chloropropane have not been shown to support growth under aerobic conditions.

Cometabolism of several short-chain halogenated aliphatics results from the lack of selectivity of some microbial enzymes. In this case, microorganisms convert a substrate without gaining energy or carbon building blocks for biosynthetic processes, and thus the organisms require the presence of a second substrate for growth. For several environmental pollutants, such as chlorinated ethenes, cometabolism is at this moment the only available option for biodegradation under aerobic conditions. Cometabolic transformations are also important for the biodegradation of recalcitrant C3-haloaliphatic compounds.

Some of the di- and trihalogenated compounds are thus far only known to be degraded by cometabolic transformations (Table 2).

Enzymatic dehalogenation of halogenated aliphatics

A critical step in a microbial pathway for the degradation of halogenated compounds is the cleavage of the carbon-halogen bond. This step may occur abiotically, but in most cases dehalogenation is enzymatically driven. As can be seen from the data in Table 2, enzymes involved in the dehalogenation of C3-haloaliphatics can be grouped in the following three categories: haloalkane dehalogenases, haloalcohol dehalogenases, and oxygenases.

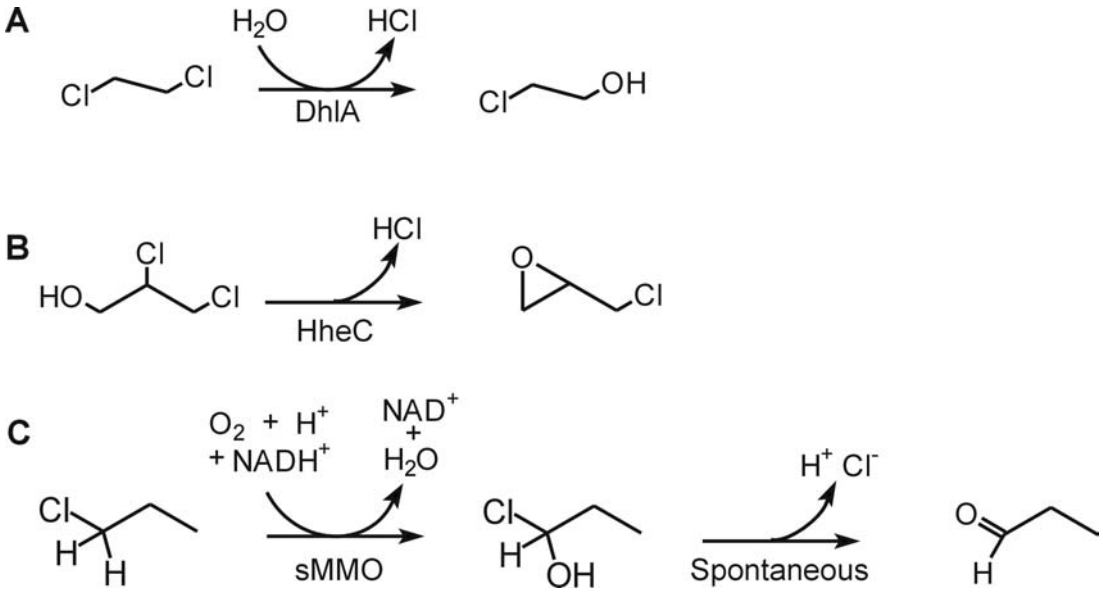

Fig. 2. (A) Hydrolytic dehalogenation catalyzed by a haloalkane dehalogenase. (B) Intramolecular substitution catalyzed by a haloalcohol dehalogenase. (C) Oxygenolytic dehalogenation catalyzed by a monooxygenase.

Haloalkane dehalogenase

Haloalkane dehalogenases are involved in the initial dehalogenation of haloalkanes, and are thereby responsible for the capability of microorganisms to degrade these compounds (40). These enzymes catalyze the hydrolytic cleavage of carbon-halogen bonds in a variety of haloalkanes (Fig. 2A). Haloalkane dehalogenases have a globular structure and are composed of two domains (49, 60, 101): a large central catalytic domain with an α/β-hydrolase fold structure, and a second domain which lies like a cap on the main domain. The latter domain emerges as a large α-helical excursion between β-strands 6 and 7 of the catalytic core. Different haloalkane dehalogenases show significant structural homology in the hydrolase core but diverge in the cap domain. The interface of the two domains defines the hydrophobic active site cavity containing the catalytic triad, which is composed of a nucleophilic aspartate, an invariant histidine and either an aspartate or a glutamate as the third member. Presently, the haloalkane dehalogenases of the strains *Xanthobacter autotrophicus* GJ10 (DhlA) (40), *Rhodococcus rhodochrous* NCIMB 13064 (DhaA) (81), and *Sphinghomonas paucimobilis* UT26 (LinB) (56) have been extensively studied. These enzymes display a broad substrate tolerance toward various chloro- and bromoalkanes, but are evolutionary optimized for the conversion of different haloalkanes. DhlA is most active on C2-C4 mono- and dihalogenated

alkanes, whereas both DhaA and LinB recognize a range of C2-C9 cyclic-, mono-, di-, and polyhalogenated alkanes. At least 25 different halogenated aliphatics have been recognized as a substrate for one or more of these enzymes, including several environmentally important chemicals such as 1,3-dichloropropene, 1,2-dibromoethane, 1,2-dibromo-3-chloropropane, and 1,2-dichloropropane.

Haloalkane dehalogenase from *R. rhodochrous* NCIMB13064 (DhaA) is the first catabolic enzyme involved in the degradation of several C_2-C_8 *n*-haloalkanes (43). The *dhaA* gene encodes a protein of 293 amino acids which shares homology with DhlA in the hydrolase core, but the proteins diverge significantly in the cap domain (60). DhaA and DhlA have conserved amino acid residues involved in catalysis indicating that they have a similar reaction mechanism. Asp106, Glu130, and His272 form the DhaA catalytic triad. Consistent with its higher activity for larger substrates, the *Rhodococcus* enzyme has a substantially larger active site cavity than DhlA (60).

An understanding of the catalytic mechanism of dehalogenases has emerged from detailed studies on the haloalkane dehalogenase from *X. autotrophicus* GJ10. These include determination of X-ray structures of reaction intermediates (102), site-directed mutagenesis of catalytic residues (42, 67-69), and mechanistic and kinetic studies (78, 79). The enzyme-catalyzed dehalogenation reaction involves the S_N2 displacement of halide by the nucleophilic carboxylate of Asp124 at the active site of the dehalogenase, forming a covalently bound ester intermediate. In the second step the ester intermediate is hydrolyzed by a water molecule that is activated by the diad His289 and Asp260. During cleavage of the carbon-halogen bond the leaving halide is stabilized by interaction with the NH's of Trp125 and Trp175. Release of the halide is the last step in the catalytic cycle.

Structural analysis of DhlA also suggested ways of engineering enzyme activity. In particular, alteration of the cap domain was found to increase the dehalogenase activity for some substrates (29, 80). However, attempts to improve the dehalogenase activity for environmentally important compounds such as DCP and TCP have been unsuccessful. Recently, with the development of rapid gene evolution technologies, there is an increasing commercial interest in engineering haloalkane dehalogenases for application in industrial processes, such as waste remediation and chemical synthesis (25, 88).

Haloalcohol dehalogenases

The haloalcohol dehalogenase reaction was discovered in 1968 by Castro and Bartnicki in a *Flavobacterium* isolated on 2,3-dibromo-1-propanol (10). Since then a number of bacteria have been isolated that produce haloalcohol dehalogenases (Table 2). These enzymes occur in the degradation pathways of halopropanols and 1,2-dibromoethane. The reaction catalyzed by a haloalcohol dehalogenase involves an intramolecular substitution of a halogen by a hydroxyl group in *vic*-halohydrins, yielding an epoxide, a proton, and a halide ion (Fig. 2B).

Haloalcohol dehalogenases have commercial applicability in bioremediation and, more important, in industrial biocatalysis (20, 36, 88). The stereoselectivity of haloalcohol

dehalogenases has allowed the development of microbial processes for the manufacture of optically active compounds, such as epichlorohydrin and glycidol (37). These compounds find application as chiral intermediates in the production of pharmaceuticals and agrochemicals. Industrial interest in haloalcohol dehalogenases has therefore focused mainly on the group of C3-halohydrins including 1,3-dichloro-2-propanol, 2,3-dichloro-1-propanol, and 3-chloro-1,2-propanediol.

Six haloalcohol dehalogenases have been described to date: the enzymes from *Corynebacterium* sp. strain N-1074 (HheA and HheB) (57), *Arthrobacter* sp. strain AD2 ($HheB_{AD2}$) (94), *Agrobacterium* sp. (23DCPOHase) (19), *Arthrobacter erithii* H10a (DehA) (2), and *Agrobacterium radiobacter* strain AD1 (HheC) (98). Haloalcohol dehalogenases are multimeric proteins composed of 2-4 subunits ranging in size from 28 to 35 kDa. The different dehalogenases are active on a range of C2, C3, and aromatic halohydrins. However, there are some distinct differences in substrate specificity and stereoselectivity between these enzymes. Whereas all haloalcohol dehalogenases described to date are active on 1,3-dichloro-2-propanol, only HheC and 23DCPOHase exhibited considerable activity on 2,3-dichloro-1-propanol.

Insight in the catalytic mechanism of haloalcohol dehalogenases has been hampered by the lack of a three-dimensional structure. Sequence analysis suggests that the overall structure of these enzymes closely resemble that of short-chain dehydrogenases/reductases (SDRs) (98). Using the coordinates of SDR family members as template, a model of a part of the HheC structure was predicted which showed considerable similarity to the X-ray structure of 7α-hydroxysteroid dehydrogenase. In the model, the position of the essential catalytic residues of 7α-hydroxysteroid dehydrogenase are conserved in HheC. Subsequent site-directed mutagenesis showed that a triad formed by the amino acid residues Ser132, Tyr145, and Arg149 was essential for catalysis in haloalcohol dehalogenase. The proposed catalytic mechanism for HheC involves proton abstraction from the hydroxyl group by Tyr145, accompanied by a nucleophilic attack of the oxyanion on the adjacent halogen-substituted carbon atom, resulting in ring closure and displacement of halide. The catalytic serine 132 plays a role in substrate binding by hydrogen bonding to the hydroxyl oxygen, whereas Arg149 may be involved in lowering the pKa of the Tyr145 causing it to be deprotonated. Haloalcohol dehalogenases constitute a unique type of dehalogenating enzymes, since these enzymes do not use a covalent mechanism for catalysis as was found for the well-studied haloalkane dehalogenases (98).

Monooxygenases

Oxidation of haloalkanes can lead to dehalogenation as a result of the spontaneous elimination of a halide from an unstable geminal halohydrin, which is generated during the oxidation of haloalkanes catalyzed by a monooxygenase (Fig. 2C). Monooxygenases are multifunctional enzymes that catalyze a range of reactions, and are normally produced to initiate the oxidation of growth-supporting substrates. Organisms producing such enzymes include bacteria that oxidize toluene, phenol, methane, propane, propylene, cumene, isoprene,

and ammonia (22). In addition, it has been shown that these bacteria often can also co-oxidize a variety of halogenated aliphatic compounds. This has led to the study and development of microbiological techniques for bioremediation of halogenated aliphatics that do not support growth of bacteria

The methane monooxygenase and ammonia monooxygenase produced by methanotrophic and nitrifying bacteria, respectively, have been shown to be active on a number of halopropanes (61, 74, 83, 100). However the conversion rates are very low and degradation is often incomplete. Methane monooxygenases have been studied in great detail. Dependent on the growth conditions a soluble or a particulate (membrane-bound) methane monooxygenase is produced. Virtually all methanotrophs can express a copper-containing membrane-bound particulate methane monooxygenase (pMMO) with a narrow substrate range (61, 97). Under conditions of low copper availability some methanotrophs can express a soluble methane monooxygenase (sMMO) that contains two iron atoms in the active site (61). The sMMO oxidizes a wider range of halogenated aliphatics than pMMO (61, 97). The sMMO proteins isolated from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b have been studied extensively (reviewed in (51) and (48) and references therein). The genes encoding the sMMO proteins have been identified and sequenced. sMMO consist of three proteins, a multimeric hydroxylase (251 kDa), a reductase (38 kDa), and a coupling protein (15.5 kDa), all three of which are required for efficient catalysis. The three-dimensional structures of the hydroxylase and the coupling protein have been solved by X-ray crystallography and NMR-spectroscopy, respectively. The hydroxylase protein is a $\alpha_2\beta_2\gamma_2$ heterodimer containing a non-heme diiron centre where dioxygen activation and methane hydroxylation occur. The reductase protein provides the electrons for the hydroxylase reaction, where they are used for the activation of O_2. The coupling protein appears to have several regulatory activities. The sMMO active site contains a hydrophobic substrate-binding pocket that can accommodate a wide variety of hydrocarbon substrates besides methane, including saturated and unsaturated, linear, branched, and cyclic hydrocarbons of up to about C8, as well as aromatic, heterocyclic, and chlorinated compounds.

The environmental relevance of sMMO is related to the wide range of reactions that it can catalyze and to its broad substrate range. This makes sMMO a promising enzyme to use for the degradation of recalcitrant environmental pollutants. However, sMMOs are complex enzymes, involving multiple subunits, and requiring a soluble cofactor. The heterologous expression of the hydroxylase protein has also proven to be problematic. Furthermore, conversion of halogenated compounds often leads to the formation of reactive products, such as acylchlorides, that may inactivate the sMMO (96). Altogether this complicates the development of treatment systems based on the activity of sMMO.

Engineering catabolic pathways for bioremediation

The appearance of bacteria that can degrade various man-made chemicals (Table 2) indicated that under selective pressure of environmental pollution, microbial capacity for the degradation of halogenated aliphatics may develop. Genetic analysis of the bacteria involved teaches us a lot about new ways to deal with yet non-degradable recalcitrant pollutants (14, 18, 50, 64). New catabolic pathways evolve through a natural genetic engineering process. This involves mutation, recombination of sequences, and transfer of DNA segments between different microbial species. These adaptation mechanisms, which apparently do occur in the environment, can in principle also be exploited under laboratory conditions in order to obtain hybrid organisms, recombined catabolic operons, or modified proteins with desired properties. The ability to mimic and accelerate natural evolution in the laboratory could shave years of the lag period between the introduction of a new synthetic compound into the environment and the development of a microbial capacity that will remove it. A number of approaches can be used to obtain organisms with the desired biodegradative capabilities.

In vivo selection

The simplest strategy is to enrich bacteria with new degradative capabilities by sustained selective pressure in chemostats, in batch enrichments, or using plate cultures. This approach does not require detailed information about the degradation mechanism, but it can take considerable time before a stable pure culture is obtained and in many cases no adaptation is found even after years. *In vivo* selection often depends on the spontaneous transfer of genetic elements encoding catabolic functions, either within a mixed population of bacteria present in the inoculum, or within the pure culture itself. Natural gene transfer events are the basis of various *in vivo* design experiments, which are greatly facilitated by the fact that many catabolic pathways for the degradation of halogenated compounds are encoded on mobile DNA elements such as conjugative plasmids or transposons. The continuous culture selection experiments of Kellogg and coworkers is an illustrative example of how the *in vivo* approach can be used to isolate microorganisms capable of utilizing highly chlorinated and persistent compounds, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), as sole source of carbon and energy (39). The synthetic organic compound 2,4,5-T has been released in massive amounts as a herbicide for weed control and was used as a defoliating agent during the Vietnam War (Agent Orange). The natural microflora degrades 2,4,5-T very slowly by co-oxidation, which results in the persistence of this toxic chemical for long periods of time in the environment. Incubation of bacteria harboring a variety of catabolic plasmids in a chemostat yielded after 8 to 10 months of selection on increasing concentrations of 2,4,5-T a pure culture of *Burkholderia cepacia* that was capable of utilizing 2,4,5-T as a sole carbon and energy source (41). The evolution of this catabolic capacity occurred most likely through recruitment of genes present in the chemostat consortium and their integration into the genome. The presence of insertion sequences adjacent to the gene clusters involved in 2,4,5-T metabolism supports this idea (18, 30).

In vivo selection has also proven to be useful for the isolation of mutants of key enzymes that constitute barriers for the metabolism of certain xenobiotics. The pioneering studies of Clarke and coworkers on the *Pseudomonas aeruginosa* amidase describe one of the classical experiments in laboratory evolution of enzymes with novel activities (12). *P. aeruginosa* can use acetamide as a growth substrate, since the amidase can hydrolyze this compound to ammonia and acetate, which can be used as a source of nitrogen and carbon, respectively. Using a variety of acetamide analogues it was possible to select many mutant *P. aeruginosa* strains that produce amidases with altered substrate specificities, which enabled the mutants to grow on aliphatic amides that cannot be utilized by the wild-type strain (12). *In vivo* selection was also successfully applied to obtain mutants of haloalkane dehalogenase DhlA with different substrate specificity. Wild-type DhlA slowly dehalogenated 1-chlorohexane, which hampered the mineralization of this compound by the recombinant strain *Pseudomonas putida* GJ31(pPJ20) expressing the *dhlA* gene. A few months of selection in batch culture yielded a number of mutant strains that could grow on 1-chlorohexane. Six different variants of DhlA were obtained with improved activity on 1-chlorohexane. All mutations were located in the cap domain, indicating an important role of this domain in determining substrate specificity of haloalkane dehalogenases (70).

In vitro construction of catabolic pathways

The possibility of using gene technology allows the rational combination of useful catabolic genes to create hybrid metabolic pathways for improving biodegradation of recalcitrant pollutants. This requires detailed genetic and biochemical information on the degradation pathways of xenobiotics. In the past decades considerable progress has been made in exploring catabolic genes, especially for halogenated aromatic compounds. Lehrbach et al. were the first who reported of an *in vitro* constructed catabolic pathway (46). Introduction of three catabolic genes cloned on a broad host range plasmid expanded the catabolic range of *Pseudomonas* sp. B13 to include 3-chlorobenzoate, 4-chlorobenzoate, and 3,5-dichlorobenzoate. In the late 1980s the design of catabolic pathways for bioremediation had a boost with a series of papers published by Timmis and coworkers (72, 76). By combining genes from different origins they created hybrid pathways that are able to eliminate combinations of synthetic compounds, such as mixtures of chloro-benzoates and alkyl-benzoates. Whereas the separate compounds are easy biodegradable, these compounds persist when they are present as mixtures due to the induction of incompatible pathways.

The engineering of bacteria for the degradation of polyhalogenated compounds is another example that demonstrates the value of recombinant DNA technology for constructing bacteria with broad metabolic capacities. These compounds are generally very resistant to biodegradation and degradation typically requires multiple mechanisms of dehalogenation. Polyhalogenated compounds are most often partly metabolized by anaerobic bacteria via reductive dehalogenation reactions. The products of these reductive dehalogenations are typically substrates for bacterial oxygenases. This necessitates the sequential use of anaerobic and aerobic bacteria for the degradation of polyhalogenated

compounds, which appears to be cumbersome from an engineering point of view. To circumvent this, both activities have been combined into a single organism (104). Cytochrome $P450_{cam}$ monooxygenase from *Pseudomonas putida* G786 and toluene dioxygenase from *Pseudomonas putida* F1 were linked and expressed in *P. putida* G786 to catalyze consecutive cometabolic reductive and oxidative dehalogenation reactions (Fig. 3). Pentachloroethane was reduced by cytochrome $P450_{cam}$ monooxygenase to trichloroethylene, and the latter product was subsequently further oxidized by toluene dioxygenase yielding formate and glycolate as final non-toxic products. In addition, the recombinant strain was also found to be active on a number of polyfluorinated, chlorinated, and brominated compounds (31).

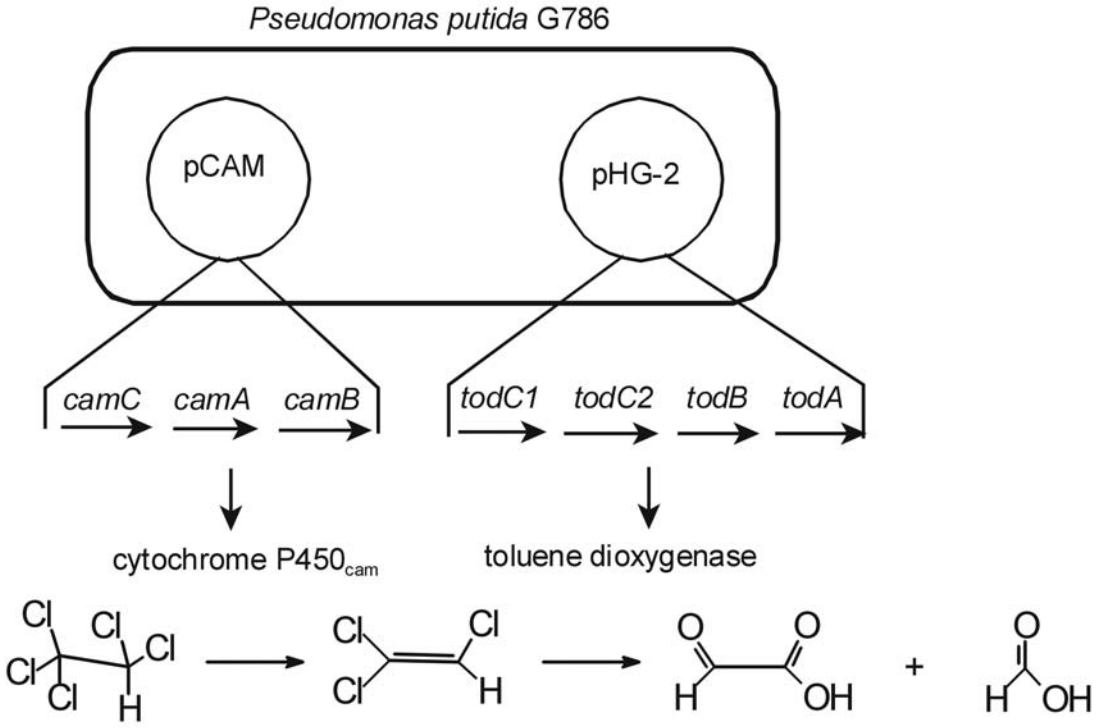

Fig. 3. Genetic construction and sequential dehalogenation reactions catalyzed by the recombinant bacterium *P. putida* G786(pHG-2). Source: Hur et al. (31)

Strategies for optimizing proteins

Protein design methods can be used to modify the selectivity and activity of catabolic enzymes, which offers opportunities for enhancing the degradation of recalcitrant compounds. Although there are many ways to evolve proteins, the strategy of most methods is to create variants and select those that perform best. In general two protein engineering strategies can be distinguished: existing enzymes can either be tailored by rational (structure-based) redesign, or by applying directed evolution methods. The latter method involves the construction of large libraries of variants using random mutagenesis or gene recombination, followed by selection or screening to identify the mutants with desired traits.

Rational design

Rational design of proteins relies on detailed knowledge of protein structure, function and mechanism, and on molecular modeling to predict favorable amino acid changes. A small number of promising modifications are introduced into the gene using site-directed mutagenesis, and the effects are tested. Rational approaches have been used recently to engineer properties of enzymes involved in the degradation of xenobiotics. For example, guided by structural data of reaction intermediates of haloalkane dehalogenase from *X. autotrophicus* GJ10, bulky amino acids lining the active site cavity were replaced by smaller residues increasing the volume of the active site. The resulting variants showed improved activity on 1,6-dichlorohexane compared to the wild type (29). In the absence of structural information, sequence comparison of related enzymes could also facilitate the choice for a particular mutational site. This was, for instance, applied to influence the substrate specificity of biphenyl dioxygenases (53). Based on the modular nature of many catabolic enzymes more drastic changes in protein function can be obtained by the construction of hybrid enzymes. Enhanced conversion rates on trichloroethylene, for instance, were obtained with hybrid enzymes created from a toluene and biphenyl dioxygenase (23). Recently, a combinatorial approach was developed for the creation of hybrid enzymes. This technique, designated ITCHY (Incremental Truncation to Create Hybrid enzymes), allows the combination of protein domains with low or no appreciable degree of homology (62).

Although rational design has proven fruitful, our insufficient understanding of enzyme-substrate interactions that contribute to catalysis impedes this approach in many cases, so that variants frequently fail to show the desired improvements. Currently, evolutionary protein design methods are the most effective approaches for improving enzyme function. These methods have been developed to such an extent that improved enzymes can be obtained on a reasonable timescale, and require little or no information regarding protein structure and function, as will be discussed below.

Random point mutagenesis

Random point mutagenesis of the gene of interest is typically performed by error-prone PCR (8), although mutator strains (5) and exposure to chemical mutagens (89) have also been used. A library of mutant enzymes, containing one or two amino acid substitutions, is created and searched with an appropriate selection or screen to identify functionally improved variants. The winner of the first round is then subjected to additional iterative rounds of random mutagenesis and selection. This process results in the accumulation of single mutations over many generations. Sequential rounds of random mutagenesis is a simple and effective strategy that has been successively used to evolve proteins with enhanced activity (35), altered substrate specificity (7), thermostability (109), solvent tolerance (54), and improved enantioselectivity (47). A large family of enzymes that might find applications in areas ranging from chemical synthesis to environmental remediation are cytochrome P450 monooxygenases. Interest in these enzymes is based on the ability to catalyze the insertion of oxygen into a wide variety of compounds, including many

xenobiotics. Using random mutagenesis and a fluorescent whole-cell screen, Arnold and coworkers identified several variants of cytochrome $P450_{CAM}$ monooxygenase from *P. putida* with high activity against naphthalene compared to the native enzyme (34, 35).

Iterated random point mutagenesis proved to be very successful in cases where most likely several sequence solutions exist and the obtained mutations are additive, for example when improving the stability of an enzyme. To evolve complex systems such as an operon, or in case that a new protein function requires the simultaneous introduction of multiple amino acid substitutions, random mutagenesis will fail. For example, despite extensive efforts no improved variants of the dibenzothiophene monooxygenase (DBT-MO) were obtained after random point mutagenesis of *dszC* genes. However, *in vitro* recombination of two homologous *dszC* genes yielded variants with higher activity and broader substrate range, most likely because recombination allows more diverse permutations of multiple beneficial mutations than random mutagenesis alone (13).

In vitro recombination

An important aspect of natural evolution that is lacking in the mutagenesis methods described above is the ability to recombine mutations from individual genes, akin to natural sexual recombination. Recombination followed by selection combines useful mutations and simultaneously flushes out harmful mutations. The view of recombination as the key accelerator of evolution has inspired the invention of *in vitro* recombination methods.

The most widely used method for *in vitro* recombination, pioneered by Stemmer, is based on random fragmentation of a pool of related DNA sequences and reassembly of the fragments into full-length genes in a primerless DNA amplification reaction (gene shuffling) (58). During this process random point mutations are introduced at a controllable rate (110). Multiple cycles of fragment reassembly results in crossovers in regions with sequence homology due to template switching creating novel combinations of mutations. Subsequent expression of the chimeric sequences followed by an effective screen or selection identifies improved variants. A pool of the best clones can then be subjected to additional rounds of DNA shuffling and selection or screening, until the evolved sequence encodes the desired function. Originally, DNA shuffling has been used for the recombination of mutations present in single gene sequences. However, the most powerful advantage of DNA shuffling is that it allows recombination of multiple parental gene sequences, which dramatically accelerates evolution of protein function. This has been nicely demonstrated for subtilisin (59). Due to their importance in the detergent industry, subtilisins have frequently been targeted in the past 30 years for improvements using rational design and random mutagenesis. Although the improvements have been notable, they are often at the expense of other properties that are critically required for industrial implementation such as thermostability, solvent stability and pH dependence (73). DNA shuffling of 26 protease genes obtained from *Bacillus* yielded several chimeric proteases with multiple improved properties, and new combinations of properties not exhibited by any of the parent enzymes (59).

DNA shuffling has been applied to a variety of enzymes to improve protein folding (16), substrate specificity (106) and enzyme activity under diverse physical and chemical conditions (24, 55, 85). In addition to improving single gene sequences, DNA shuffling can also address more complex problems, such as optimizing an entire biodegradative pathway. The arsenate operon of *Staphylococcus aureus*, for example, was evolved by three rounds of DNA shuffling. Cells expressing the optimized operon were able to grow on 0.5 M arsenate, corresponding to a 40-fold increase in resistance (15). Furthermore, whole cells can be improved by recombining entire genomes (108).

Table 3. Examples of directed evolution of enzymes involved in xenobiotic degradation

Enzyme	Property	Change effected	Approach	Ref.
Biphenyl dioxygenases	Substrate specificity	Accepts novel PCB congeners. Enhanced degradation of benzene, toluene, and alkylbenzene.	DNA shuffling of homologous genes + screening	(44, 87)
Arsenate detoxification pathway	Arsenic resistance	40-fold improvement in arsenate resistance	DNA shuffling + selection	(15)
Atrazine chlorohydrolase	Substrate specificity	Widening of the substrate range, up to 150 –fold greater conversion rates	DNA shuffling of two homologous genes + screening	(71, 82)
Toluene dioxygenase	Substrate specificity	Higher activity on a novel substrate 4-picoline, improved toluene conversion	Error-prone PCR, saturation mutagenesis + screening	(77)
Toluene *ortho*-monooxygenase	Degradation of chlorinated compounds	Improved conversion of chlorinated ethylenes	DNA shuffling + screening	(9)
2-Hydroxybiphenyl 3-monooxygenase	Substrate specificity	Improved activity on fungicide 2-hydroxybiphenyl Increased activity on novel substrates	Error-prone PCR + screening	(52)
Cytochrome P450 monooxygenase	Catalytic activity	20-fold higher activity on naphthalene	Error-prone PCR followed by recombination + screening	(35)
Haloalkane dehalogenase DhlA	pH-optimum	lower pH optimum	Mutator strain + screening	(11)
Haloalkane dehalogenase DhaA	Thermostability	30,000-fold more stable than the parent	Gene site saturation mutagenesis + screening	(25)

Although application of evolutionary protein design methods has been mainly focused on improving enzymes for industrial biocatalysis, there is an increasing interest in evolving enzymes for bioremediation. Table 3 summarizes a number of enzymes involved in the degradation of xenobiotics which have been improved using directed evolution.

At the moment evolutionary methods are the preferred strategy for protein engineering, however, the capabilities of rational design methods, particularly computational techniques, are expanding too (103). Interestingly, directed evolution technology is now increasingly applied to investigate structure-function relationships of proteins (84).

The aim of this thesis

Although numerous halogenated aliphatics can serve as a bacterial growth substrate, there are still many of these compounds that are resistant towards biodegradation. Moreover, a number of these recalcitrant compounds are of industrial and environmental importance. The primary challenge of bioremediation is therefore to develop strategies to tackle these recalcitrant compounds. The work described in this thesis is focused on TCP, which was based on considerable TCP groundwater pollution at a former dumping site of chemical waste (92). Mineralization of TCP in a bioreactor system would be the most elegant way to deal with this pollutant. For 1,2-dichloroethane, a compound that has similar physico-chemical properties as TCP, a full-scale groundwater treatment system employing *X. autotrophicus* GJ10 has been developed (86). This indicates that the acquisition of bacterial cultures that can degrade TCP is of critical importance. However, preliminary experiments indicated that TCP is very recalcitrant towards biodegradation.

The primary goal of this work was therefore to obtain bacterial growth on the chlorinated hydrocarbon 1,2,3-trichloropropane. Comparative studies on the bacterial degradation of chlorinated aliphatics that are structurally similar to 1,2,3-trichloropropane indicate that the initial dehalogenation of 1,2,3-trichloropropane is the critical step. In chapter two and three we investigated two systems which can catalyze this first step. In **Chapter 2** we describe the cometabolic conversion of chloropropanes by whole cells of *Methylosinus trichosporium* OB3b expressing the soluble methane monooxygenase (sMMO). The relevance of sMMO is that it can oxidize a wide range of compounds including chlorinated aliphatic compounds. Enzymatic conversion of a range of halogenated propanes by haloalkane dehalogenase from *Rhodococcus* sp. DhaA is described in **Chapter 3**. The enzyme exhibited activity on 1,2,3-trichloropropane yielding 2,3-dichloro-1-propanol. The pathway of dehalogenation by DhaA was established using rapid-quench flow analysis of 1,3-dibromopropane conversion. The results define a minimal kinetic mechanism consisting of four main steps. In **Chapter 4** we describe the design of a catabolic pathway for trihalopropanes by introducing broad-host-range *dhaA* expression plasmids into *Agrobacterium radiobacter* AD1, which has the ability to utilize dihalogenated propanols for growth. The recombinant strain AD1(pTB3), expressing the *dhaA* gene under the control of the *dhlA* promoter, was able to utilize both 1,2,3-tribromopropane and 1,2-dibromo-3-chloropropane as sole carbon sources. Although 1,2,3-trichloropropane was converted by the recombinant strain, the activity of DhaA on 1,2,3-trichloropropane appeared to be insufficient to sustain bacterial growth. The work presented in **Chapter 5** focuses on improving the catalytic activity of DhaA on 1,2,3-trichloropropane by random mutagenesis. A double mutant of DhaA exhibited an improved activity on TCP. Degradation of TCP by a recombinant strain expressing the evolved dehalogenase was evaluated. Furthermore, modeling of the evolved enzyme revealed some insight in the molecular basis for the improved dehalogenase activity. Finally, in **Chapter 6** the results are summarized and discussed.

1. **Alexander, M.** 1995. How toxic are toxic chemicals in soil. Environ. Sci. Technol. **29:**2713-2717.
2. **Assis, H. M. S., P. J. Sallis, A. T. Bull, and D. J. Hardman.** 1998. Biochemical characterization of a haloalcohol dehalogenase from *Arthrobacter erithii* H10a. Enz. Microbiol. Technol. **22:**568-574.
3. **Babich, H. D., L. Davis, and G. Stotzky.** 1981. Dibromochloropropane (DBCP): a review. Sci. Total Environ. **17:**207-221.
4. **Belkin, S.** 1992. Biodegradation of haloalkanes. Biodegradation. **3:**299-313.
5. **Bornscheuer, U. T., J. Altenbuchner, and H. H. Meyer.** 1998. Directed evolution of an esterase for the stereoselective resolution of a key intermediate in the synthesis of epothilones. Biotechnol. Bioeng. **58:**554-559.
6. **Bosma, T., E. Kruizinga, E. J. de Bruin, G. J. Poelarends, and D. B. Janssen.** 1999. Utilization of trihalogenated propanes by *Agrobacterium radiobacter* AD1 through heterologous expression of the haloalkane dehalogenase from *Rhodococcus* sp. strain m15-3. Appl. Environ. Microbiol. **65:**4575-4581.
7. **Bruhlmann, F., and W. Chen.** 1999. Tuning biphenyl dioxygenase for extended substrate specificity. Biotechnol. Bioeng. **63:**544-551.
8. **Cadwell, R. C., and G. F. Joyce.** 1994. Randomization of genes by PCR mutagenesis. PCR methods and applications. **2:**28-33.
9. **Canada, K. A., S. Iwashita, H. Shim, and T. K. Wood.** 2002. Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. J. Bacteriol. **184:**344-349.
10. **Castro, C. E., and E. W. Bartnicki.** 1968. Biodehalogenation: Epoxidation of halohydrins, epoxide opening, and transhalogenation by a *Flavobacterium* sp. Biochemistry **7:**3213-3218.
11. **Chang, C. H., J. F. Schindler, C. J. Unkefer, L. A. Vanderberg, J. R. Brainard, and T. C. Terwilliger.** 1999. In vivo screening of haloalkane dehalogenase mutants. Bioorg. Med. Chem. **7:**2175-2181.
12. **Clarke, P. H.** 1978. Experiments in microbial evolution., p. 137-218. *In* L. N. Ornston and J. R. Sokatch (ed.), The Bacteria, vol. 4. Academic, New York.
13. **Coco, W. M., W. E. Levinson, M. J. Crist, H. J. Hektor, A. Darzins, P. T. Pienkos, C. H. Squires, and D. J. Monticello.** 2001. DNA shuffling method for generating highly recombined genes and evolved enzymes. Nat. Biotechnol. **19:**354-359.
14. **Copley, S. D.** 2000. Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. Trends in Biochem. Sci. **25:**261-265.
15. **Crameri, A., G. Dawes, E. Rodriguez, Jr., S. Silver, and W. P. Stemmer.** 1997. Molecular evolution of an arsenate detoxification pathway by DNA shuffling. Nat. Biotechnol. **15:**436-438.
16. **Crameri, A., E. A. Whitehorn, E. Tate, and W. P. C. Stemmer.** 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat. Biotechnol. **14:**315-319.
17. **Curragh, H., O. Flynn, M. J. Larkin, T. M. Stafford, J. T. Hamilton, and D. B. Harper.** 1994. Haloalkane degradation and assimilation by *Rhodococcus rhodochrous* NCIMB 13064. Microbiol. **140:**1433-1442.
18. **Daubaras, D. L., C. E. Danganan, A. Hubner, R. W. Ye, W. Hendrickson, and A. M. Chakrabarty.** 1996. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* strain AC1100: Evolutionary insight. Gene **179:**1-8.
19. **Effendi, A. J., S. D. Greenaway, and B. N. Dancer.** 2000. Isolation and characterization of 2,3-dichloro-1-propanol degrading *Rhizobia*. Appl. Environ. Microbiol. **66:**2882-2887.
20. **Fauzi, A. M., D. J. Hardman, and A. T. Bull.** 1996. Biodehalogenation of low concentrations of 1,3-dichloro-2-propanol by mono- and mixed cultures of bacteria. Appl. Microbiol. Biotechnol. **46:**660-666.
21. **Fetzner, S.** 1998. Bacterial dehalogenation. Appl. Microbiol. Biotechnol. **50:**633-657.
22. **Fetzner, S., and F. Lingens.** 1994. Bacterial dehalogenases: Biochemistry, Genetics, and Biotechnological applications. Microbiol. Rev. **58:**641-685.
23. **Furukawa, K., J. Hirose, S. Hayashida, and K. Nakamura.** 1994. Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase. J. Bacteriol. **176:**2121-2123.
24. **Giver, L., A. Gershenson, P. O. Freskgard, and F. H. Arnold.** 1998. Directed evolution of a thermostable esterase. Proc. Natl. Acad. Sci. USA **95:**12809-12813.
25. **Gray, K. A., T. H. Richardson, K. Kretz, J. M. Short, F. Bartnek, R. Knowles, L. Kan, P. E. Swanson, and D. E. Robertson.** 2001. Rapid evolution of reversible denaturation and elevated melting temperature in a microbial haloalkane dehalogenase. Adv. Synth. Cat. **343:**607-617.
26. **Gribble, G. W.** 1998. Naturally occurring organohalogen compounds. Acc. Chem. Res. **31:**141-152.
27. **Hartmans, S., and J. A. De Bont.** 1992. Aerobic vinyl chloride metabolism in Mycobacterium aurum L1. **58:**1220-1226.
28. **Health Council of the Netherlands. Dutch Expert Committee on Occupational Standards (DECOS),** 1994. 1,2,3-Trichloropropane. Report no. 1994/25. Health Council of the Netherlands, The Hague, The Netherlands.
29. **Holloway, P., K. L. Knoke, J. T. Trevors, and H. Lee.** 1998. Alteration of the substrate range of haloalkane dehalogenase by site-directed mutagenesis. Biotechnol. Bioeng. **59:**520-523.
30. **Hubner, A., C. E. Danganan, L. Y. Xun, A. M. Chakrabarty, and W. Hendrickson.** 1998. Genes for 2,4,5-trichlorophenoxyacetic acid metabolism in *Burkholderia cepacia* AC1100: Characterization of the *tftC* and *tftD* genes and locations of the *tft* operons on multiple replicons. Appl. Environ. Microbiol. **64:**2086-2093.
31. **Hur, H. G., M. J. Sadowsky, and L. P. Wackett.** 1994. Metabolism of chlorofluorocarbons and polybrominated compounds by *Pseudomonas putida* G786(pHG-2) via an engineered metabolic pathway. Appl. Environ. Microbiol. **60:**4148-4154.
32. **Imai, R., Y. Nagat, K. Senoo, H. Wada, M. Fukuda, M. Takagi, and K. Yano.** 1989. Dehydrochlorination of γ-hexachlorocyclohexane (γ-BHC) by γ-BHC-assimilating *Pseudomonas paucimobilis*. Agric. Biol. Chem. **53:**2015-2017.
33. **Janssen, D. B., A. Scheper, L. Dijkhuizen, and B. Witholt.** 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49:**673-677.
34. **Joo, H., A. Arisawa, Z. Lin, and F. H. Arnold.** 1999. A high-throughput digital imaging screen for the discovery and directed evolution of oxygenases. Chem. Biol. **6:**699-706.
35. **Joo, H., Z. Lin, and F. H. Arnold.** 1999. Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. Nature **399:**670-673.

36. **Kasai, N., T. Suzuki, and Y. Furukawa.** 1998. Chiral C3 epoxides and halohydrins: Their preparation and synthetic application. J. Mol. Cat. B:Enz. **4:**237-252.
37. **Kasai, N., K. Tsujimura, K. Unoura, and T. Suzuki.** 1992. Isolation of (*S*)-2,3-dichloro-1-propanol assimilating bacterium, its characterization, and its use in preparation of (*R*)-2,3-dichloro-1-propanol and (*S*)-epichlorohydrin. J. Ind. Microbiol. **10:**37-43.
38. **Kasai, N., T. Tsujimura, K. Unoura, and T. Suzuki.** 1990. Degradation of 2,3-dichloro-1-propanol by a *Pseudomonas* sp. Agric. Biol. Chem. **54:**3185-3190.
39. **Kellogg, S. T., D. K. Chatterjee, and A. M. Chakrabarty.** 1981. Plasmid-assisted molecular breeding: new technique for enhanced biodegradation of persistent toxic chemicals. Science **214:**1133-1135.
40. **Keuning, S., D. B. Janssen, and B. Witholt.** 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. **163:**635-639.
41. **Kilbane, J. J., D. K. Chatterjee, J. S. Karns, S. T. Kellog, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. **44:**72-78.
42. **Krooshof, G. H., E. M. Kwant, J. Damborsky, J. Koca, and D. B. Janssen.** 1997. Repositioning the catalytic triad aspartic acid of haloalkane dehalogenase: effects on stability, kinetics, and structure. Biochemistry **36:**9571-9580.
43. **Kulakova, A. N., M. J. Larkin, and L. A. Kulakova.** 1997. The plasmid-located haloalkane dehalogenase gene from *Rhodococcus rhodochrous* NCIMB 13064. Microbiol. **143:**1-7.
44. **Kumamaru, T., H. Suenaga, M. Mitsuoka, T. Watanabe, and K. Furukawa.** 1998. Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. Nat. Biotechnol. **16:**663-666.
45. **Lage, M., J. G. Omichinski, E. Dybing, S. D. Nelson, and E. J. Soderlund.** 1994. Mutagenic activity of halogenated propanes and propenes: effect of bromine and chlorine positioning. Chem. Biol. Interact. **93:**73-84.
46. **Lehrbach, P. R., J. Zeyer, W. Reineke, H. J. Knackmuss, and K. N. Timmis.** 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* sp. strain B13. J. Bacteriol. **158:**1025-1032.
47. **Liebeton, K., A. Zonta, K. Schimossek, M. Nardini, D. Lang, B. W. Dijkstra, M. T. Reetz, and K. E. Jaeger.** 2000. Directed evolution of an enantioselective lipase. Chem. Biol. **7:**709-718.
48. **Lipscomb, J. D.** 1994. Biochemistry of the soluble methane monooxygenase. Annu. Rev. Microbiol. **48:**371-399.
49. **Marek, J., J. Vevodova, I. K. Smatanova, Y. Nagata, L. A. Svensson, J. Newman, M. Takagi, and J. Damborsky.** 2000. Crystal structure of the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26. Biochemistry **39:**14082-14086.
50. **Martinez, B., J. Tomkins, L. P. Wackett, R. Wing, and M. J. Sadowsky.** 2001. Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. J. Bacteriol. **183:**5684-5697.
51. **Merkx, M., D. A. Kopp, M. H. Sazinsky, J. L. Blazyk, J. Muller, and S. J. Lippard.** 2001. Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: A tale of two irons and three proteins. Angew. Chem. Int. Ed. **40:**2782-2807.
52. **Meyer, A., A. Schmid, M. Held, A. H. Westphal, M. Rothlisberger, H. P. Kohler, W. J. van Berkel, and B. Witholt.** 2001. Changing the substrate reactivity of 2-hydroxybiphenyl 3-monooxygenase from *Pseudomonas azelaica* HBP1 by directed evolution. J. Biol. Chem. **277:**5575-5582.
53. **Mondello, F. J., M. P. Turcich, J. H. Lobos, and B. D. Erickson.** 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl. Environ. Microbiol. **63:**3096-3103.
54. **Moore, J. C., and F. H. Arnold.** 1996. Directed evolution of a para-nitrobenzyl esterase for aqueous-organic solvents. Nat. Biotechnol. **14:**458-467.
55. **Moore, J. C., H. M. Jin, O. Kuchner, and F. H. Arnold.** 1997. Strategies for the in vitro evolution of protein function: enzyme evolution by random recombination of improved sequences. J. Mol. Biol. **272:**336-347.
56. **Nagata, Y., K. Miyauchi, J. Damborsky, K. Manova, A. Ansorgova, and M. Takagi.** 1997. Purification and characterization of a haloalkane dehalogenase of a new substrate class from a γ-hexachlorocyclohexane-degrading bacterium, *Sphingomonas paucimobilis* UT26. Appl. Environ. Microbiol. **63:**3707-3710.
57. **Nakamura, T., T. Nagasawa, F. Yu, I. Watanabe, and H. Yamada.** 1992. Resolution and some properties of enzymes involved in enantioselective transformation of 1,3-dichloro-2-propanol to (*R*)-3-chloro-1,2-propanediol by *Corynebacterium* sp. strain N-1074. J. Bacteriol. **174:**7613-7619.
58. **Ness, J. E., S. B. Del Cardayre, J. Minshull, and W. P. C. Stemmer.** 2001. Molecular breeding: The natural approach to protein design. Adv. Prot. Chem. **55:**261-292.
59. **Ness, J. E., M. Welch, L. Giver, M. Bueno, J. R. Cherry, T. V. Borchert, W. P. C. Stemmer, and J. Minshull.** 1999. DNA shuffling of subgenomic sequences of subtilisin. Nat. Biotechnol. **17:**893-896.
60. **Newman, J., T. S. Peat, R. Richard, L. Kan, P. E. Swanson, J. A. Affholter, I. H. Holmes, J. F. Schindler, T. C. Unkefer, and T. C. Terwilliger.** 1999. Haloalkane dehalogenases: Structure of a *Rhodococcus* enzyme. Biochemistry **38:**16105-16114.
61. **Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt.** 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55:**2819-2826.
62. **Ostermeier, M., J. H. Shim, and S. J. Benkovic.** 1999. A combinatorial approach to hybrid enzymes independent of DNA homology. Nat. Biotechnol. **17:**1205-1209.
63. **Pilorz, B. H.** 1962. Allyl chloride and derivatives., p. 714-715. *In* J. S. Sconce (ed.), Chlorine: Its manufacture, properties and uses. Reinhold Publishing Corp., New York, N. Y.
64. **Poelarends, G. J., L. A. Kulakov, M. J. Larkin, J. E. van Hylckama Vlieg, and D. B. Janssen.** 2000. Roles of horizontal gene transfer and gene integration in evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways. J. Bacteriol. **182:**2191-2199.
65. **Poelarends, G. J., J. E. van Hylckama Vlieg, J. R. Marchesi, L. M. Freitas Dos Santos, and D. B. Janssen.** 1999. Degradation of 1,2-dibromoethane by *Mycobacterium* sp. strain GP1. J. Bacteriol. **181:**2050-2058.
66. **Poelarends, G. J., M. Wilkens, M. J. Larkin, J. D. van Elsas, and D. B. Janssen.** 1998. Degradation of 1,3-dichloropropene by *Pseudomonas cichorii* 170. Appl. Environ. Microbiol. **64:**2931-6.

67. **Pries, F., J. Kingma, and D. B. Janssen.** 1995. Activation of an Asp-124->Asn mutant of haloalkane dehalogenase by hydrolytic deamidation of asparagine. FEBS Lett. **358:**171-174.
68. **Pries, F., J. Kingma, G. H. Krooshof, C. M. Jeronimus Stratingh, A. P. Bruins, and D. B. Janssen.** 1995. Histidine 289 is essential for hydrolysis of the alkyl-enzyme intermediate of haloalkane dehalogenase. J. Biol. Chem. **270:**10405-10411.
69. **Pries, F., J. Kingma, M. Pentenga, G. van Pouderoyen, C. M. Jeronimus Stratingh, A. P. Bruins, and D. B. Janssen.** 1994. Site-directed mutagenesis and oxygen isotope incorporation studies of the nucleophilic aspartate of haloalkane dehalogenase. Biochemistry **33:**1242-1247.
70. **Pries, F., A. J. van den Wijngaard, R. Bos, M. Pentenga, and D. B. Janssen.** 1994. The role of spontaneous cap domain mutations in haloalkane dehalogenase specificity and evolution. J. Biol. Chem. **269:**17490-17494.
71. **Raillard, S., A. Krebber, Y. C. Chen, J. E. Ness, E. Bermudez, R. Trinidad, R. Fullem, C. Davis, M. Welch, J. Seffernick, L. P. Wackett, W. P. C. Stemmer, and J. Minshull.** 2001. Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. Chem. Biol. **8:**891-898.
72. **Ramos, J. L., A. Wasserfallen, K. Rose, and K. N. Timmis.** 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science **235:**593-596.
73. **Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande.** 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. **62:**597-635.
74. **Rasche, M. E., R. E. Hicks, M. R. Hyman, and D. J. Arp.** 1990. Oxidation of monohalogenated ethanes and *n*-chlorinated alkanes by whole cells of *Nitrosomonas europaea*. J. Bacteriol. **172:**5368-5373.
75. **Rieger, P. G., H. M. Meier, U. Vogt, T. Groth, and H. J. Knackmuss.** 2002. Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence. J. Biotechnol. **94:**101-123.
76. **Rojo, F., D. H. Pieper, K.-H. Engesser, H.-J. Knackmuss, and K. N. Timmis.** 1987. Assemblage of ortho-cleavage route for simultaneous degradation of chloro-and methylaromatics. Science **238:**1395-1398.
77. **Sakamoto, T., J. M. Joern, A. Arisawa, and F. H. Arnold.** 2001. Laboratory evolution of toluene dioxygenase to accept 4-picoline as a substrate. Appl. Environ. Microbiol. **67:**3882-3887.
78. **Schanstra, J. P., and D. B. Janssen.** 1996. Kinetics of halide release of haloalkane dehalogenase: evidence for a slow conformational change. Biochemistry **35:**5624-5632.
79. **Schanstra, J. P., J. Kingma, and D. B. Janssen.** 1996. Specificity and kinetics of haloalkane dehalogenase. J. Biol. Chem. **271:**14747-14753.
80. **Schanstra, J. P., A. Ridder, J. Kingma, and D. B. Janssen.** 1997. Influence of mutations of Val226 on the catalytic rate of haloalkane dehalogenase. Prot. Eng. **10:**53-61.
81. **Schindler, J. F., P. A. Naranjo, D. A. Honaberger, C.-H. Chang, J. R. Brainard, L. A. Vanderberg, and C. J. Unkefer.** 1999. Haloalkane dehalogenases: Steady-state kinetics and halide inhibition. Biochemistry **38:**5772-5778.
82. **Seffernick, J. L., and L. P. Wackett.** 2001. Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. Biochemistry **40:**12747-12753.
83. **Shimoda, M., Y. Seki, and I. Okura.** 1993. Oxidation of halogenated propanes with *Methylosinus trichosporium* OB3b. J. Mol. Catal. **83:**L5-L10.
84. **Spiller, B., A. Gershenson, F. H. Arnold, and R. C. Stevens.** 1999. A structural view of evolutionary divergence. Proc. Natl. Acad. Sci. USA **96:**12305-12310.
85. **Stemmer, W. P.** 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature **370:**389-391.
86. **Stucki, G., and M. Thuer.** 1995. Experiences of a large-scale application of 1,2-dichloroethane degrading microorganisms for groundwater treatment. Environ. Sci. Technol. **29:**2339-2345.
87. **Suenaga, H., M. Mitsuoka, Y. Ura, T. Watanabe, and K. Furukawa.** 2001. Directed evolution of biphenyl dioxygenase: emergence of enhanced degradation capacity for benzene, toluene, and alkylbenzenes. J. Bacteriol. **183:**5441-5544.
88. **Swanson, P. E.** 1999. Dehalogenases applied to industrial-scale biocatalysis. Curr. Opin. Biotechnol. **10:**365-369.
89. **Taguchi, S., A. Ozaki, T. Nonaka, Y. Mitsui, and H. Momose.** 1999. A cold-adapted protease engineered by experimental evolution system. J. Biochem. (Tokyo). **126:**689-693.
90. **Tesoriero, A. J., F. E. Loffler, and H. Liebscher.** 2001. Fate and origen of 1,2-dichloropropane in an unconfined shallow aquifer. Environ. Sci. Technol. **35:**455-461.
91. **United States Environmental Protection Agency.** NationalUnited States Priority Site Fact Sheet. http://www.epa.gov/region02/superfund/npl/ciba/. Last updated on October, 2001.
92. **United States Environmental Protection Agency**. Tysons Dump Site. http://www.epa.gov/reg3hwmd/super/PA/tysons-dump/index.htm.Last updated on October, 1999.
93. **van Agteren, M. H., S. Keuning, and D. B. Janssen.** 1998. Handbook on biodegradation and biological treatment of hazardous organic compounds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
94. **van den Wijngaard, A. J., P. T. W. Reuvekamp, and D. B. Janssen.** 1991. Purification and characterization of haloalcohol dehalogenase from *Arthrobacter* strain AD2. J. Bacteriol. **173:**124-129.
95. **van der Ploeg, J., J. Kingma, E. J. de Vries, J. G. M. van der Ven, and D. B. Janssen.** 1996. Adaptation of *Pseudomonas* sp. GJ1 to 2-bromoethanol caused by overexpression of an NAD-dependent aldehyde dehydrogenase with low affinity for halogenated aldehydes. Arch. Microbiol. **165:**258-264.
96. **van Hylckama Vlieg, J. E. T., W. de Koning, and D. B. Janssen.** 1997. Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **63:**4961-4964.
97. **van Hylckama Vlieg, J. E. T., W. de Koning, and D. B. Janssen.** 1996. Transformation kinetics of chlorinated ethenes by *Methylosinus trichosporium* OB3b and detection of unstable epoxides by on-line gas chromatography. Appl. Environ. Microbiol. **62:**3304-3312.
98. **van Hylckcama Vlieg, J. E. T., L. X. Tang, J. H. L. Spelberg, T. Smilda, G. J. Poelarends, T. Bosma, A. E. J. van Merode, M. W. Fraaije, and D. B. Janssen.** 2001. Halohydrin dehalogenases are structurally and mechanistically related to short-chain dehydrogenases/reductases. J. Bacteriol. **183:**5058-5066.
99. **van Rijn, J. P., N. M. van Straalen, and J. Willems.** 1995. Handboek bestrijdingsmiddelen: gebruik en milieu-effecten. p. 42. VU uitgeverij, Amsterdam. The Netherlands.
100. **Vannelli, T., M. Logan, D. M. Arciero, and A. B. Hooper.** 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. **56:**1169-1171.

101. **Verschueren, K. H., S. M. Franken, H. J. Rozeboom, K. H. Kalk, and B. W. Dijkstra.** 1993. Refined X-ray structures of haloalkane dehalogenase at pH 6.2 and pH 8.2 and implications for the reaction mechanism. J. Mol. Biol. **232:**856-872.
102. **Verschueren, K. H., F. Seljee, H. J. Rozeboom, K. H. Kalk, and B. W. Dijkstra.** 1993. Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase. Nature **363:**693-698.
103. **Voigt, C. A., S. L. Mayo, F. H. Arnold, and Z. G. Wang.** 2001. Computational method to reduce the search space for directed protein evolution. Proc. Natl. Acad. Sci. USA **98:**3778-3783.
104. **Wackett, L. P., M. J. Sadowsky, L. M. Newman, H. G. Hur, and S. Li.** 1994. Metabolism of polyhalogenated compounds by a genetically engineered bacterium. Nature **368:**627-629.
105. **Wijngaard, A. J. v. d., D. B. Janssen, and B. Witholt.** 1989. Degradation of epichlorohydrin and halohydrins by bacteria isolated from fresh water sediment. J. Gen. Microbiol. **135:**2199-2208.
106. **Yano, T., S. Oue, and H. Kagamiyama.** 1998. Directed evolution of an aspartate aminotransferase with new substrate specificities. Proc. Natl. Acad. Sci. USA **95:**5511-5515.
107. **Yokota, T., F. Hiroyuki, T. Omori, and Y. Minoda.** 1986. Microbial dehalogenation of haloalkanes mediated by oxygenases or halidohydrolase. Agric. Biol. Chem. **50:**453-460.
108. **Zhang, Y.-X., K. Perry, V. A. Vinci, K. Powell, W. P. C. Stemmer, and S. B. del Cardayre.** 2002. Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature **415:**644-646.
109. **Zhao, H., and F. H. Arnold.** 1999. Directed evolution converts subtilisin E into a functional equivalent of thermitase. Prot. Eng. **12:**47-53.
110. **Zhao, H., and F. H. Arnold.** 1997. Optimization of DNA shuffling for high fidelity recombination. Nucleic. Acids. Res. **25:**1307-1308